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CELLULAR MIXED DISULPHIDES BETWEEN THIOLS AND PROTEINS, AND THEIR POSSIBLE IMPLICATION FOR RADIATION PROTECTION

H. MODIG

Department of Tumor Biology, Karolinska Institutet Medical School, Stockholm 60, Sweden

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Abstract—The occurrence of mixed disulphides between thiols and proteins has been demonstrated in crystalline bovine serum albumin and in proteins of Ehrlich ascites tumour cells. The thiols could be released from the proteins by reduction of the disulphide bonds with sodium borohydride. On the average, 25 per cent of the released thiols was glutathione. It is suggested that protein bound glutathione may represent a reservoir of physiological radiation protector. The release of glutathione from cellular proteins by an exchange reaction may be of significance for the radioprotective action of cysteamine.

THE MECHANISM of action of sulphydryl containing radioprotective compounds is still subject to considerable controversy[1]. A temporary binding of radioprotective thiols to proteins by the formation of disulphide bonds has been demonstrated, among other, by Eldjarn and Pihl[2], Kollmann and Shapiro[3] and Betz *et al.*[4]. Formation of such transient mixed disulphides has been proposed to explain the radioprotective action observed, by defending the —SH groups of proteins from an irreversible binding with radiation induced radicals.[5] It has not yet been established with certainty, whether such mixed disulphides occur in the cell under physiological conditions, but many observations do, in fact, indicate that this may be the case.

Huisman and Dozy[6] have demonstrated the presence of a particular peptide when digests of a fraction of human hemoglobin were subjected to electrophoretic analysis. This peptide was subsequently identified as glutathione. Eagle and co-workers[7] have shown that L-cystine is essential for the growth of mouse and human cells in tissue culture medium. They reported, however, that a number of different thiols, disulphides and inorganic reducing agents which contain sulphur, were able to promote cell growth even in a cystine-deficient medium. It was suggested that these substances were not used for cystine synthesis, but acted by releasing half-cystine residues which are bound in the form of mixed disulphides to the serum proteins of the medium. Mize *et al.*[8] have obtained evidence that a mixed disulphide exists between glutathione and the enzyme glutathione reductase in rat liver cells. The binding of glutathione to

The following abbreviations will be used throughout this paper: BSA: bovine serum albumin; CySH: cysteamine; DTNB: 5,5′ dithiobis-2-nitrobenzoic acid; GSH: glutathione; GSSG: oxidized glutathione; $NADPH_2$: reduced nicotinamide-adenindinucleotide-phosphate; NPSH: non-protein bound sulphydryl groups; NPSS: non-protein bound disulphides; PSH: protein bound sulphydryl groups; PSSP: protein bound disulphide groups; PSSNP and PSSG: mixed disulphides between protein and non-protein sulphydryls and protein and glutathione, respectively; Tris: Tris(hydroxymethyl)aminomethane.

cellular enzymes has also been shown by Krimsky and Racker[9] who reported that heat or trypsin treatment of purified glyceraldehyde-3-dehydrogenase will release glutathione from the protein. Similarly, Register *et al.*[10] found that incubation of liver homogenates with crystalline trypsin almost doubled the amount of sulphydryl in the homogenate.

Bovine serum albumin is reported to contain 0·67 —SH groups/mole.[11] This amount is increased to 1·0 —SH group/mole upon treatment with β-mercaptoethanol or urea. King[12] has proposed that the difference i.e. 0·33 —SH group/mole, is engaged in the formation of disulphide bonds between cysteine and protein on the one hand and on the other hand, to a lesser extent, between glutathione and protein. This view has also been supported by Hartley and co-workers,[13] who found a non-sulphydryl containing protein-fraction when they carried out chromatographic studies on serum albumins. This fraction could be converted into —SH containing albumin after treatment with β-mercaptoethanol. The —SH groups were considered to have been hidden in disulphide bonds with low molecular thiols. Recently, McArdle[14] has demonstrated the presence of reactive intermolecular disulphide bonds between bovine serum non-mercaptalbumin and two low molecular thiols by reduction with sulphite and subsequent gel-filtration. The amount of released thiols was in the order of 1·0 mole of —SH/mole of protein, and behaved chromatographically in a manner similar to glutathione and cysteine.

It was demonstrated by Révész and Modig[15] that incubation of Ehrlich ascites tumour cells in a tissue-culture medium which contained increasing concentrations of cysteamine, produced a gradual increase of the intracellular level of non-protein bound sulphydryl groups. About 40 per cent of the NPSH increase was found to be due to a rise of the glutathione level in the cells. Release of glutathione from cellular mixed disulphides with proteins by an exchange reaction with cysteamine was considered as a possible explanation for this finding expressed by the formula:

$$PSSG + CySH \rightarrow PSSCy + GSH$$

It was suggested that the rise of the cellular content of glutathione might be of significance for the radioprotective action of cysteamine. As an extension of the previous investigation, this paper reports studies performed with the purpose to confirm the presence of physiologically occurring mixed disulphides in mammalian cells and, furthermore, to characterize them more closely, with regard to the extent that they are present in the cells, and to examine, thus their possible significance for natural and induced radiation protection. Experiments with bovine serum albumin, having a well defined molecular weight and a known number of —SH and disulphide groups, were included into the investigation as particular controls in order to establish the accuracy of the methods used.

MATERIALS

An Ehrlich ascites tumour, denoted ELD, was used as cell material. It was maintained by weekly intraperitoneal transfers of 2×10^6 cells in hybrid mice. In all experiments freshly withdrawn tumour which had grown for 5–7 days was used. Samples with blood-contamination were discarded. An electronic particle counter (Coulter Counter Model B) was used for cell counts.

Bovine serum albumin (Cohn fraction V) was obtained from Armour Pharmaceutical Company, Eastbourne. Trizma Base, obtained from Sigma Chemical Co., was used for the preparation of Tris-buffers. Buffer solutions were made, in all cases, in demineralized water and bubbled through with 0_2-free nitrogen gas (100 ppm) for 5 min to diminish auto-oxidation of —SH groups. Enzyme-preparations, glyoxalase-I (sp. act. 300 I.E./mg) and glutathione reductase (specific activity 90 I.E./mg) were purchased from C. F. Boehringer & Soehne, Mannheim. All other chemicals used were of highest purity commercially obtainable.

METHODS

Quantitative estimation of cellular sulphydryl groups

The cells in 1 ml ascites fluid ($150–200 \times 10^6$) were washed twice in 20 vol. of a balanced salt solution by gentle centrifugation at 500 *g*. The concentration of non-protein bound sulphydryl groups in the cells was determined according to the method of Ellman.[16] The DTNB-reagent was added to a sulfosalicylic acid extract of the cells, after adjustment of the solution to pH 6·8 with 1 M $NaHCO_3$ and 0·2 M phosphate buffer. The absorbance was read at 412 mμ, and the concentration of —SH calculated with reference to a standard glutathione curve. The S.E. of a single determination as established from 20 duplicate measurements of the —SH content of the average cell was 1·9 per cent of the mean.

Glutathione concentration was determined specifically by the glyoxalase-method,[17] which measures the formation of lactyl-S-glutathione from methyl-glyoxal in the presence of glyoxalase-I and GSH, as revealed by the increase of absorbance at 240 mμ. It was found in model experiments, that this method is highly specific for GSH even in the presence of a 10-fold concentration of cysteine or cysteamine. The standard error of a determination, established from 12 duplicate measurements, was 5·3 per cent of the mean.

Determination of protein bound sulphydryl groups was carried out by an automatic amperometric titration procedure, based upon the method of Benesch *et al.*[18]. A platinum electrode, connected to the circuit by a mercury column, and rotated by a synchronous motor at 1500 r.p.m. was used. As a reference electrode, the Hg-HgO-saturated-$Ba(OH)_2$ half cell of Samuelson and Brown[19] was employed. It was connected to the titration cell by a saturated KCl-filled glass-bridge, fitted with a porous glass filter at its end. A counter-current of 2·5 mA was inserted into the circuit for use as reference potential. Titration was performed with 5×10^{-3} M $AgNO_3$, delivered into the titration cell by an Agla microsyringe. The micrometer of the latter was attached to a synchronous motor which revolved at 1 r.p.m. and delivered, thus, 0·05 μmole $AgNO_3$/min. The potential changes which arose in the course of titration, were recorded graphically by a potentiometer (Nesco Instruments Inc.). The endpoint of the titration was estimated by determining the intersection of the reference line and the straight part of the excess reagent line. The distance between the starting point and the endpoint was proportional to the —SH content of the sample to be titrated. The S.E. of a single determination, as established from 12 duplicate measurements of PSH in the average cell was less than 2 per cent of the mean.

Reduction of disulphide bonds

The amount of acid soluble, low molecular disulphide was determined by electro-

lytic reduction of the sulfosalicylic acid cell extract on a mercury surface according to Dohan and Woodward,[20] followed by measurement of the reduced —SH groups according to the Ellman method.[16] A current of 3·16 mA/cm^2 mercury surface was used. The S.E. of a single determination as estimated from 12 duplicate measurements was 7·2 per cent of the mean.

In certain experiments, acid soluble disulphides were also assayed enzymatically[17] with glutathione reductase. The decrease in the concentration of $NADPH_2$ in the presence of the cell extract, as revealed by the absorbance at 340 mμ, was measured. Pihl *et al.*[21] showed that glutathione reductase reduces GSSG as well as other low molecular disulphides in the presence of small amount of GSH. The difference in the values obtained by the enzymatic method and the method of Dohan and Woodward amounted to about 25 per cent of the mean.

For reduction of protein bound disulphide bonds, the proteins of a known number of washed tumour cells were precipitated with ice-cold 6 per cent perchloric acid. The precipitate was washed 3 times in 10 vol. of the acid in order to remove acid soluble thiols and disulphides. Subsequently, the precipitate was dissolved in Tris-buffer (pH 7·4) to a final concentration of about 1 per cent. Insoluble material was removed by centrifugation in a Spinco Ultracentrifuge for 30 min ($g = 25{,}000$). The clear supernatant contained about 70–80 per cent of the original amount of cellular proteins as determined by the method of Lowry *et al.*[22]. In the experiments with BSA, the protein was dissolved in Tris-buffer in a concentration of 10 mg/ml. Urea was added in a concentration of 8 M as a denaturating agent. Aliquots of 10 ml of the protein solution was then transferred into 50 ml flat-bottomed glass-vials submerged and agitated in a waterbath at 40°. Sodium borohydride ($NaBH_4$), 20 mg/ml, was added as the reducing agent together with a few drops of octyl alcohol in order to prevent excessive foaming.

Reducing of disulphide bonds in proteins with sodium borohydride was first introduced by Edman and Diehl[23] and by Moore *et al.*[25] in investigations on the structure of insulin and ribonuclease, respectively. The method has later been employed for the determination of disulphide bonds in various other proteins such as fibrin and fibrinogen,[25] beef muscle protein[26] and bovine serum albumin.[27, 28] It has been demonstrated by Fashold *et al.*[29] that the disulphide bonds exclusively are attacked by the sodium borohydride reduction. In this study the reduction of disulphide bonds with $NaBH_4$ was preferred instead of the more commonly used procedures, e.g. reduction with mercaptans or sulphite, in order to avoid introduction of irrelevant —SH groups into the system which may represent a source of error in the determination of the thiol part of the mixed disulphides.

Thirty minutes was allowed for the reduction process. It was found (Fig. 1) that maximum yield of NPSH could be obtained during this period. With concentrations of urea lower than 8 M, the reduction was incomplete and the yield of NPSH decreased (Fig. 2). The amount of released GSH was almost independent of urea concentration. This may indicate that the disulphide bonds between glutathione and the proteins might be more accessible to reduction, and not dependent upon the unfolding of the protein molecules by urea treatment.

After reduction, an aliquot of the protein solution was precipitated with an equal volume of 20 per cent perchloric acid. The precipitate was centrifuged at $g = 25{,}000$ for 30 min, and the clear supernatant analyzed, on the one hand, with respect to the

concentration of the total acid soluble thiols by the non-specific Ellman method[16] and, on the other hand, with respect to GSH by the specific glyoxalase method.[17]

In another aliquot the pH was adjusted to 7·0 with 2 N HCl in order to destroy excess of $NaBH_4$ which might interfere with —SH determinations. The total amount

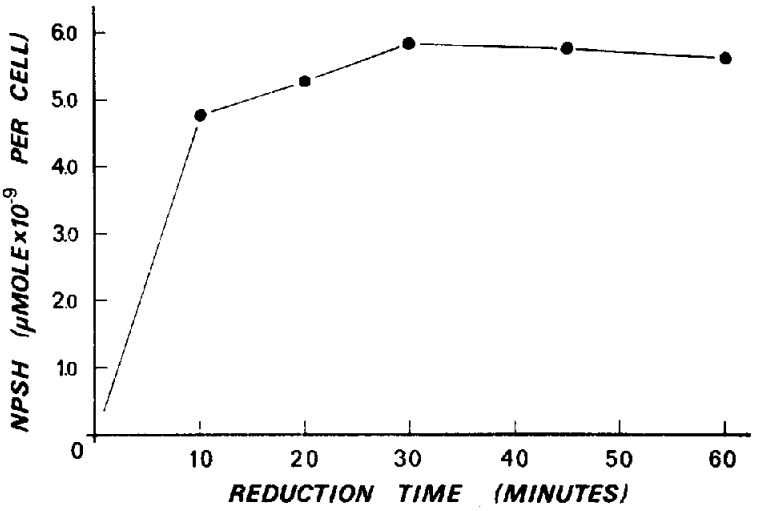

FIG. 1. Yield of NPSH from cellular proteins after different periods of reduction by $NaBN_4$ in the presence of 8 M urea.

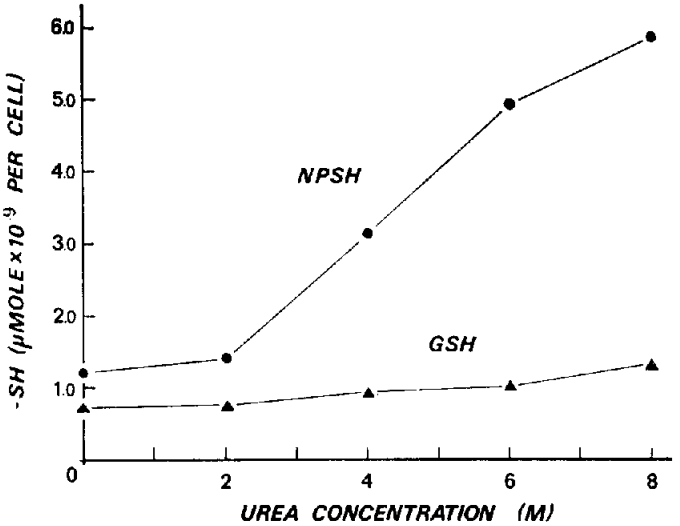

FIG. 2. Yield of NPSH and GSH from cellular proteins in the presence of varying concentrations of urea after 20 min reduction by $NaBH_4$.

of free —SH, both acid soluble and protein bound, was then determined by amperometric titration with $AgNO_3$. The amount of PSH was calculated by substracting the amount of the acid soluble sulphydryl groups, determined by the DTNB-method,[16] from the total.

RESULTS

The molar amount of PSH, NPSH and GSH in bovine serum albumin and the proteins from Ehrlich ascites tumour cells, as determined before and after treatment with $NaBH_4$ are presented in Table 1. The amounts are expressed on a molar basis in the case of BSA, and on a per cell or on a weight basis in the case of the Ehrlich ascites cell proteins. The molecular weight of BSA was taken as 67,000.

The —SH content of the BSA sample was found to be 0·65 mole/mole, when it was dissolved in Tris-buffer in the absence of urea. This value was increased by about 20 per cent (col. 4) upon dissolving of BSA in the presence of 8 M urea. After treatment with $NaBH_4$, the amount of protein bound —SH increased to 36·4 mole. It can be calculated that this increase corresponds to the reduction of 17 disulphide bonds/mole, an amount corresponding to that reported to occur in BSA[30] (cp. col. 7). The amount of NPSH released upon reduction was found to be in the order of 1 mole/mole of BSA. In agreement with Andersson.[31] 0·16 mole of the released thiols were found to consist of glutathione. The nature of the remaining portion of the released NPSH was not determined. It can be considered to consist of half-cystine residues.[14, 31]

The amount of protein bound —SH groups in the cellular proteins was $16{\cdot}71 \times 10^{-9}$ μmole/cell. Upon reduction with $NaBH_4$, this value was increased nearly 3-fold, corresponding to the reduction of about 12–13 micromole of protein bound disulphide groups (col. 7). The amount of released thiols which can be considered to occur bound to the cellular proteins as mixed disulphides, was found to be $5{\cdot}91 \times 10^{-9}$ μmole per cell. This value is approximately twice the normal ($2{\cdot}46 \times 10^{-9}$) free, non-protein bound thiol content of the cell. The normal thiol content is due almost exclusively to glutathione.[15] Glutathione constituted 24 per cent of the released thiols. This implies that about 35 per cent of the total cellular glutathione content exists in a protein bound form. The nature of the non-glutathione portion of NPSH has not been established. Analogously to the finding made with bovine serum albumin, it may consist of cysteine residues.[14, 31]

When expressed on a mg protein basis the relative proportion of released NPSH and GSH to PSH, determined in separate experiments, is in agreement with the findings expressed on a per cell basis. The data permit the calculation of the amount of mixed disulphides in the protein molecules investigated. In BSA, the mixed disulphides can be calculated to amount to about 6 per cent of the total disulphide bonds. In the cellular proteins, the corresponding figure is about 30 per cent.

DISCUSSION

The results indicate that a considerable part of the cellular thiols may be bound to proteins in the form of mixed disulphides. These thiols may be regarded as a cellular reservoir of physiological protective agent which can be utilised in a situation when, for some reason, the cellular level of NPSH is reduced. There is some indirect evidence that such a situation may occur and that protein bound thiols are released intracellularly. After irradiation of tumour cells, in the presence of oxygen, the rapid intracellular formation of NPSS in excess to the NPSH decrease indicates that newly liberated NPSH must participate in the disulphide formation.[32] The mixed disulphides may conceivably represent a source of the excess formation of NPSS.

Formation of GSH in excess of that expected to occur when all the available GSSG

TABLE 1. AMOUNT OF —SH GROUPS IN BOVINE SERUM ALBUMIN AND EHRLICH ASCITES TUMOUR CELL PROTEINS BEFORE AND AFTER TREATMENT WITH $NaBH_4$ IN THE PRESENCE OF UREA

Type of protein	No. experiments	Before $NaBH_4$ treatment	After $NaBH_4$ treatment				
				NPSH			
		PSH	PSH	GSH	Total	PSSP§	PSSNP¶
1	2	3	4	5	6	7	8
Bovine serum albumin	12	0·78 ± 0·09*	36·4 ± 0·37*	0·16 ± 0·02*	1·02 ± 0·08*	17·3 ± 0·19*	5·9
Cellular proteins	11	16·71 ± 0·39†	47·20 ± 1·71†	1·40 ± 0·05†	5·91 ± 0·29†	12·34 ± 0·63†	32·4
	6	9·80 ± 0·64‡	26·03 ± 1·28‡	0·64 ± 0·04‡	2·53 ± 0·21‡	6·85 ± 0·53‡	27·0

* Mole —SH/mole BSA ± S.E.
† μMole —SH × 10^{-9} per cell ± S.E.
‡ μMole —SH × 10^{-3}/mg protein ± S.E.
§ Calculated from values in cols. 3, 4 and 6.
¶ As percent of the total amount of disulphide bonds, calculated from values in cols. 6 and 7.

would be reduced, was noted after incubation of tumour cells in a medium supplemented with cysteamine.[15]

Mercaptan—disulphide interchange reactions, reported to occur in pure chemical,[33] as well as in biological[34] systems, can be considered to explain this finding. The changed balance between PSH and NPSH which follows upon the cellular introduction of cysteamine may initiate reversible reactions between the free thiols on the one hand, and protein bound —SH and disulphides on the other[2–4]. This would result in the binding of cysteamine and the release of protein bound thiols. The possible significance of the protein binding of the introduced protector has recently been extensively discussed by Gorin.[35]

Considering such interchange reactions it is conceivable that the radioprotective effect afforded by cysteamine may be due to an intracellular release of NPSH. More specifically, in view of our data which indicate that a considerable portion of the released NPSH consists of GSH, the effect of cysteamine may be attributed to the liberation of GSH. Accordingly, cysteamine may exert its protective effect essentially by strengthening the physiological radiation protection afforded by the free glutathione present in the cell.[36] The possible identity of the released GSH with the hypothetical substance YSH, proposed by Bacq,[1] as has been suggested recently,[37] has yet to be established.

Glutathione has been found a protector with moderate effect *in vivo*,[1] even though it is active *in vitro*.[38] The difficult penetration of the molecule through the cell membrane may explain this difference. When released inside the cell, it may, however, exert a powerful protection. Considering the molecular weight of GSH, which is about 3 times of that of cysteine and cysteamine, it may more efficiently scavenge radiation induced radicals at equal molar concentrations. Using pulse radiolysis technique, it has been shown that glutathione has the highest rate constant in reactions with OH· radicals in comparison to a number of other thiols.[39] Glutathione is also known to specifically eliminate radiation induced hydrogen peroxides via the glutathione peroxidase pathway.[40] The function of glutathione as a prosthetic group to many essential enzymes[9, 41] may also be of importance in connection to the repair of radiation injury.

The equivocal interpretations which suggest that, on the one hand, low molecular disulphides amount to less than 10 per cent[42, 43] and, on the other hand, to about 50 per cent[44] of the total amount of low molecular —SH in mammalian cells can be explained by considering the intracellular occurence of mixed disulphides between thiol and proteins.

In the case when large amounts of NPSS were suggested to occur,[44] the method of disulphide determination involved treatment of the supernatant of cellular aqueous homogenates with $NaBH_4$. This procedure can be considered to reduce, in addition to NPSS, also the mixed disulphides of the dissolved proteins. If the calculation of the amount of low molecular disulphides is based solely on the amount of NPSH found after this type reduction, disregarding that a part of it may be released from mixed disulphides, it is obvious that incorrectly high values will be obtained. On the other hand, when reduction is carried out after precipitation of the proteins,[42, 43] the correct values should be expected.

Results of experiments carried out with the purpose of testing the validity of this consideration, are presented in Table 2. The NPSS content of Ehrlich ascites tumour cells was determined by electrolytic,[20] enzymatic[17] and $NaBH_4$ reduction of acidic

extracts of the tumour cells, and by $NaBH_4$ reduction of the supernatant of aqueous cell homogenates. Similar estimates were obtained amounting to about 10 per cent of the total amount of low molecular sulphydryls in all cases, with the exception when reduction concerned also the dissolved cellular proteins. In that case, the estimate

TABLE 2. CONCENTRATION OF LOW MOLECULAR, ACID SOLUBLE DISULPHIDES IN EHRLICH ASCITES TUMOUR CELLS, AS ESTIMATED BY DIFFERENT METHODS

NPSS	Electrolytic reduction	Enzymatic reduction	$NaBH_4$ reduction	
			Before de-proteinization	After de-proteinization
μMole $\times 10^{-9}$ per cell $\pm$ S.E.	0·15 $\pm$ 0·02 (32)	0·20 $\pm$ 0·06 (10)	0·62 $\pm$ 0·11 (8)	0·12 $\pm$ 0·06 (8)
μMole $\times 10^{-2}$ /g wet wt. $\pm$ S.E.	1·15 $\pm$ 0·15 (32)	1·54 $\pm$ 0·46 (10)	4·77 $\pm$ 0·85 (8)	0·92 $\pm$ 0·31 (8)

Figures in parenthesis indicate the number of separate determinations.

was larger by a factor of about 4, which supports the above explanation that the inconsistency in the reported results may be due to NPSH release from mixed disulphides.

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REFERENCES

1. Z. M. BACQ, *Chemical protection against ionizing radiation*. Thomas, Springfield (1965).
2. L. ELDJARN and A. PIHL, *J. biol. Chem.* **223**, 341 (1956).
3. G. KOLLMAN and B. SHAPIRO, *Radiat. Res.* **27**, 474 (1966).
4. E. H. BETZ, D. J. MEWISSEN and P. LELIEVRE, *Int. J. Radiat. Biol.* **4**, 231 (1962).
5. L. ELDJARN and A. PIHL, *Progress in Radiology*. Oliver-Boyd, London (1956).
6. T. H. J. HUISMAN and A. M. DOZY, *J. Lab. clin. Med.* **60**, 302 (1962).
7. H. EAGLE, V. I. OYAMA and K. A. PIEZ, *J. biol. Chem.* **198**, 721 (1960).
8. C. E. MIZE, T. E. THOMPSON and R. G. LANGDON, *J. biol. Chem.* **237**, 1956. (1962).
9. I. KRIMSKY and E. RACKER, *J. biol. Chem.* **198**, 721 (1952).
10. U. D. REGISTER, A. M. LASORSA snd D. M. KATSUYAMA, *Fedn Proc.* **15**, 336 (1956).
11. R. BENESCH and R. E. BENESCH, *Fedn Proc.* **15**, 218 (1956).
12. T. P. KING, *J. biol. Chem.* **236**, PC 5 (1961).
13. R. W. HARTLEY, E. A. PETERSON and H. A. SOBER, *Biochemistry* **1**, 60 (1962).
14. C. MCARDLE, *Biochem. J.* **102**, 211 (1967).
15. L. RÉVÉSZ and H. MODIG, *Nature, Lond.* **207**, 430 (1965).
16. G. L. ELLMAN, *Archs Biochem. Biophys.* **82**, 70 (1959).
17. H. KLOTSCH and H. U. BERGMEYER, in *Methoden der enzymatischen Analyse*, (Ed. H. U. BERGMEYER) p. 363. Verlag Chemie, Weinheim (1962).
18. R. E. BENESCH, H. A. LARDY and R. BENESCH, *J. biol. Chem.* **216**, 663 (1955).
19. G. J. SAMUELSON and D. J. BROWN, *J. Am. chem. Soc.* **57**, 2711 (1935).
20. C. J. DOHAN and G. E. WOODWARD, *J. biol. Chem.* **129**, 393 (1939).
21. A. PIHL, L. ELDJARN and J. BREMER, *J. biol. Chem.* **227**, 339 (1957).
22. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FAIR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
23. P. EDMAN and K. DIEHL, 2nd *Int. Congr. Biochem.* 51, Réseumés, Paris (1952).

24. S. MOORE, R. D. COLE, G. GUNDLACH and W. H. STEIN, *Proc. 4th Int. Congr. Biochem., Vienna*, **8**, 52 (1958).
25. A. HENSCHEN, *Arkiv Kemi* **22**, 355 (1964).
26. R. HAMM and K. HOFMANN, *Nature, Lond.* **207**, 1269 (1965).
27. W. D. BROWN, *Biochem. biophys. Acta* **44**, 363 (1960).
28. D. CAVALLINI, M. T. GRAZIANI and S. DUPRÉ, *Nature, Lond.* **212**, 294 (1966).
29. H. FASOLD, G. GUNDLACH and F. TURBA, *Biochem. Z.* **334**, 255 (1961).
30. A. N. GLAZER and E. L. SMITH, *J. biol. Chem.* **236**, 416 (1961).
31. L. O. ANDERSSON, *Biochem. biophys. Acta* **117**, 115 (1965).
32. L. RÉVÉSZ and H. MODIG, *Annls Med.. exp. Biol. Fenn.* **44**, 333 (1966).
33. L. ELDJARN and A. PIHL, *J. Am. Chem. Soc.* **79**, 4589 (1957).
34. A. PIHL and L. ELDJARN, *Proc. 4th Int. Congr. Biochem.* Vienna, **8**, 43 (1958).
35. H. GORIN, *Progr. biochem. Pharmac.* **1**, 142 (1965).
36. L. RÉVÉSZ, H. BERGSTRAND and H. MODIG, *Nature, Lond.* **198**, 1275 (1963).
37. Z. M. BACQ, *Bull. Acad. r. Méd. Belg.* VII Série, **6**, 115 (1966).
38. E. S. G. BARRON, S. DICKMAN, J. A. MUNTZ and T. P. SINGER, *J. gen. Physiol.* **32**, 537 (1949).
39. G. E. ADAMS, J. W. BOAG, J. CURRANT and B. D. MICHAEL, in *Pulse Radiolysis*, p. 131. Academic Press, London (1965).
40. G. COHEN and P. HOCHSTEIN, *Biochemistry* **2**, 1420 (1963).
41. S. H. CHANG and D. R. WILKEN, *J. biol. Chem.* **241**, 4251 (1966).
42. B. SÖRBO, *Archs. Biochem. Biophys.* **98**, 342 (1962).
43. H. MARTIN and H. MCILWAIN, *Biochem. J.* **71**, 275 (1959).
44. G. S. TARNOWSKI, R. K. BARCLAY, I. M. MOUNTAIN, N. NAKAMURA, H. G. SATTERWHITE and E. M. SOLNEY, *Archs. Biochem. Biophys.* **110**, 210 (1965).